

Methotrexate polyglutamate levels in circulating erythrocytes and polymorphs correlate with clinical efficacy in rheumatoid arthritis

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Abstract Objectives

To measure MTX polyglutamates in circulating erythrocytes (E-MTX), mononuclear cells (MNC-MTX) and polymorphs (PMN-MTX) in rheumatoid arthritis (RA) patients and to see whether these correlated with clinical efficacy and side effects.

Methods

Sixty-five patients (40F, 25M; mean age 57 yrs.) with RA (ARA revised criteria) who had been on weekly pulse MTX (2.5 - 37.5 mg) for at least 2 months were entered into this study. The patients were classified as responders (R), partial responders (PR) or non-responders (NR) when blood was sampled for the MTX determination. Side effects since the initiation of MTX were also recorded. MTX-polyglutamates were measured (blinded to clinical details) using an enzymatic assay.

Results

E-MTX in responders and partial responders were significantly higher ($p < 0.001$) than in non-responders. Similarly, PMN-MTX were also higher, but the difference was only significant for the R group ($p = 0.0019$). The differences in concentrations could not be explained on the basis of the dose, which tended to be higher in NR than in R ($p = 0.085$). The concomitant prednisolone dose was significantly lower in R than in NR ($p = 0.001$), as were the ESR and CRP ($p = 0.007$, and $p = 0.05$ respectively), but the MCV was higher ($p = 0.047$). E-MTX tended to be higher in patients with side effects, but this difference did not reach statistical significance ($p = 0.15$).

Conclusion

The results suggest that circulating intracellular levels of MTX polyglutamates in RBC and PMN correlate with clinical efficacy but not with toxicity in patients with RA.

Key words

Methotrexate polyglutamates, erythrocytes, polymorphs, rheumatoid arthritis, clinical efficacy.

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Introduction

Methotrexate (MTX) is now widely used in the treatment of rheumatoid arthritis (RA) and increasingly in other chronic inflammatory conditions (1). For RA methotrexate is administered weekly in low doses, usually between 7.5 - 20 mg (1-3), although some patients fall outside this range for reasons that are unclear. While the factors determining an individual's response to the drug or the development of side effects are still not completely understood, differences in dosage requirements are usually attributed to individual differences in pharmacokinetics. Many factors are known to influence the pharmacokinetics of low dose methotrexate, including intrinsic factors such as age, renal function and variability in absorption from the bowel, and extrinsic factors such as the co-administration of methotrexate with food or other drugs (4-6).

For a number of drugs (e.g. cyclosporin A, phenytoin, digoxin, and others) a therapeutic plasma drug concentration has been defined, and plasma level measurement offers a useful tool to monitor the dosage used to achieve therapeutic efficacy and avoid side effects. On the other hand attempts to correlate measurements of the circulating levels of methotrexate in the plasma with the clinical response following low-dose therapy have not been successful. This is not surprising since using standard techniques concentrations in the plasma are measurable for less than 24 hours after the administration of the low weekly doses used in RA, while the onset and end of their effects are usually delayed by a matter of weeks (7).

One possible explanation for the delay in the onset and end of the drug's therapeutic effect may be that the activity of low dose methotrexate depends on the level of tissue accumulation either of the parent compound or of an active metabolite and that the measurement of these may be more relevant than the measurement of plasma MTX levels. Although the mechanism of action of methotrexate in RA and other chronic inflammatory conditions is not known (8), its basic action of inhibiting folate metabolism is also shared by its polyglutamate metabolites, which are stored in tissues in-

cluding the liver, in circulating erythrocytes (9) and to a lesser extent in circulating leucocytes (10).

When methotrexate is taken up by these cells it is bound intracellularly to dihydrofolate reductase and is metabolized to polyglutamate forms (11) and they, like MTX, are potent inhibitors of dihydrofolate reductase (12). Whereas unchanged methotrexate flows out of cells relatively rapidly, the intracellular polyglutamate form persists and may lead to the prolonged inhibition of dihydrofolate reductase.

In erythrocytes, the half-life of the polyglutamate formed is about four to six weeks, far greater than that of unchanged MTX (13). MTX is incorporated in the red blood cells (RBC) during erythropoiesis and is retained in the form of MTX polyglutamates in the circulating RBC pool, with a slow turnover (14). After 6 to 8 weeks of an unchanged weekly dose of MTX, a steady state erythrocyte MTX level (E-MTX) is reached (15). Since E-MTX may reflect bone marrow exposure to MTX, this may turn out to be a useful parameter for monitoring and adjusting the dose of MTX, paralleling what is incorporated into and retained by the cells, rather than monitoring plasma levels to which the cells are only transiently exposed.

Methotrexate exists in two forms in the neutrophils as well, i.e. unchanged MTX and the polyglutamate form. The latter, which is slowly diffusable, has been shown to be incorporated into myeloid cells in the bone marrow and reaches a peak concentration in circulating neutrophils at about 7 days after a 24 hr MTX infusion (16). This is the average time it takes for cells in the proliferating myeloid compartment to mature and be released into the circulation as neutrophils (17).

Similarly, it has been shown that lymphoblasts in bone marrow samples obtained from patients with acute lymphoblastic leukemia (ALL) also have the ability to accumulate MTX and MTX polyglutamates (18). In childhood acute lymphoblastic leukemia the concentration of methotrexate polyglutamates in erythrocytes is a predictor of relapse, with higher concentrations being associated with a better prognosis (19).

With this background, methotrexate polyglutamate levels in circulating erythrocytes and leucocytes were measured in rheumatoid arthritis patients to test whether these correlated with the clinical response and side effects.

Patients and methods

Patients

To be eligible for the study, patients had to meet the revised criteria of the American Rheumatism Association for rheumatoid arthritis (20), and had to have been receiving weekly pulse methotrexate for at least 2 months. The dose of methotrexate and that of other anti-rheumatic drugs had to have been stable for at least one month.

Patient characteristics recorded at the time of entry into the study included age, sex, seropositivity for rheumatoid factor, methotrexate dose, and the dose of any other anti-rheumatic or other drugs being taken at the time of study.

Clinical and disease status assessment

The same treating rheumatologist classified each patient as a responder, partial responder or non-responder at the time of the study. His evaluation was based on a physician's global clinical assessment which included the patient history, joint examination and a comparison with the patient's condition at an earlier point in time, i.e., a comparative temporal assessment. The physician was blinded to the results of the measurements of acute phase reactants and methotrexate levels (see below). The treating clinician also recorded any side effects with a definite or probable relationship to methotrexate experienced since beginning MTX. If more than one assessment was recorded for a particular patient, these had to be at least one month apart and the dose of MTX and corticosteroid had to be stable for that preceding month. Other measures recorded at the time of the study included a full blood count (Coulter counter), ESR (Westergren), C-reactive protein (CRP; Beckman Assay), liver function tests (gamma-glutamyl transferase (GT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total globulin and serum albumin) (Kodak Ektachem) and red cell folate (Ciba-Corning ACS-180 analyzer).

Intracellular methotrexate polyglutamates

At the time of each assessment an additional 20 ml of whole blood was collected into a heparinized tube for the MTX polyglutamate determination. Sampling was made on the day of, but prior to the next weekly dose when MTX was undetectable in the serum (3). Red cells, mononuclear cells (MNC) (predominantly lymphocytes) and polymorphonuclear leucocytes (PMN) were separated within 24 hours of their collection and prepared for an enzymatic analysis of the MTX polyglutamate concentration. The assayist was blinded to each patient's clinical status and other measures.

The fresh blood (20 ml) collected in lithium heparin tubes was spun down to obtain the plasma, which was stored in polypropylene tubes at -20°C . After removal of the plasma, the blood was diluted with an equal volume of Hank's balanced salt solution (C.S.L, Melbourne, Australia), layered onto Ficoll-paque (Pharmacia, Uppsala, Sweden), and centrifuged at 1750 rpm for 20 minutes at 18° to 20°C .

The mononuclear cell fraction (lymphocytes and monocytes) was removed from the buffer Ficoll interface and was washed twice with phosphate buffered saline (PBS) for 10 minutes at 1500 rpm. After removal of the buffer, mononuclears and Ficoll-paque, 3 ml of 6% Dextran T-500 (Pharmacia, Uppsala, Sweden) and 10 ml of PBS were added to the red cell-neutrophil layer and the tube was left to stand at room temperature for 12 - 15 minutes.

The neutrophil layer was removed and spun at 1750 rpm for 15 minutes, and then purified via erythrocyte hypotonic lysis to obtain more than 98% neutrophils, which were 99% viable as determined by trypan blue staining. Both the neutrophils and mononuclear cells were resuspended in 1 ml PBS and stored at -20°C until analysis.

The remaining RBCs left after the plasma, PMNs and MNCs were removed, were washed three times with ice cold 0.9% NaCl and prepared for enzymatic analysis.

The method used was adopted from both Imbert *et al.* and Schroder *et al.* (21, 22).

The erythrocytes were washed twice with ice cold 0.9% NaCl and then haemolyzed in 3 volumes of water. The haemosylate was boiled for 7 minutes, centrifuged at $9000 \times g$ for 15 min, and the clear supernatant stored at -20°C until analysed. Before analysis the PMNs and MNC were thawed, sonicated for 30 sec, boiled for 7 min, and centrifuged at $9000 \times g$ 15 min, and the clear supernatant was used for the MTX measurements.

Aqueous and haemosylate MTX standards (0, 6.25, 12.5, and 25 $\mu\text{g/L}$) were prepared from MTX powder (Sigma Chemical Co., St. Louis, Mo., USA) dissolved in water and in haemosylate (1 vol of blood to 3 vol of water), respectively. The working reagent was prepared from 7 ml of Tris HCl (0.5 mol/L, pH 6.5), 3.5 ml of KCl (1.5 mol/L), 0.85 mg NADPH (tetrasodium salt, Sigma), 10 μl of bovine dihydrofolate reductase suspension (EC 1.5.1.3, Sigma) and 15 mg bovine serum albumin (Sigma). The start reagent consisted of 25 mg of dihydrofolic acid suspended in 5 ml of 5 mmol/L HCl and 5 ml of 0.1 mmol/L β -mercaptoethanol, which was stored in 200 μl aliquotes at -20°C . Before use each 200 μl vial was diluted to 2 ml with tris HCl (0.5 mol/L, pH 7.2).

The method was adapted for a Cobas Bio centrifugal analyser in which the following settings were employed: sample volume 20 μl , diluent volume 10 μl , reagent volume 125 μl , incubation time 60 sec, temperature 25°C , start reagent volume 20 μl . The instrument measured the absorbance at 340 nm 10 and 100 sec after the addition of the starting reagent and calculated the change of absorbance in E/min.

Statistical analyses

All data are expressed as means \pm SD. Comparisons of the results between two groups were made using either the unpaired Student's t-test for normally distributed data or the Mann-Whitney U test for data that were not normally distributed. Side effect rates were compared using the 3 x 2 chi-square test. Correlations between the MTX levels and continuous parameters were examined by linear regression analysis. A P value < 0.05 was considered to indicate statistical significance.

Table I. Concurrent medications for rheumatoid arthritis in the different response groups.

Drugs	Response Category		
	Responders (n = 58)	Partial responders (n = 28)	Non-responders (n = 11)
Prednisolone	29 (50%)	14 (50%)	9 (82%)
Other second line agents (hydroxychloroquine, sulphasalazine)	34 (59%)	16 (57%)	8 (73%)
NSAIDs (ibuprofen, naproxen, diclofenac, ketoprofen, piroxicam)	20 (34%)	9 (32%)	5 (45%)
Folic acid	3 (5%)	2 (7%)	0

Results

Patient characteristics

Sixty-five patients, 40 females and 25 males (age 56.5 ± 11.3 yrs.; range 27 to 84 yrs.) with rheumatoid arthritis, who had been on weekly pulse MTX (2.5 - 37.5 mg) for at least 2 months were enrolled in the study. All were taking the drug as a single weekly oral dose except one who received MTX by IM injection. Four patients were omitted from the study for the following reasons: one patient misunderstood a dosage change and inadvertently took an excessive dose of MTX, while the remaining 3 patients were not classified according to their clinical response.

Patients in this study were also receiving one or more other agents that are commonly combined with MTX for the treatment of rheumatoid arthritis. These included prednisolone, other second-line agents (hydroxychloroquine, sulphasalazine), NSAIDs (ibuprofen, naproxen, diclofenac, ketoprofen, piroxicam), and folic acid. Table I gives the number of patients on concurrent medication in the

different response groups.

Since some of the patients were evaluated on more than one occasion, at least a month apart, the number of data sets exceeded the total number of patients. Hence, a total of 99 classification episodes were recorded for the 61 patients. These included 59 classifications as responders, 29 as partial responders and 11 as non-responders at the times of measurement of intracellular methotrexate polyglutamate concentrations.

Drug response

There was a trend for non-responders to be on a higher dose of MTX than both the responders and partial responders. At the times of assessment the weekly dose of MTX was 9.18 ± 4.85 mg in the responders, 9.83 ± 4.17 mg in the partial responders and 11.14 ± 4.79 mg in the non-responders. The difference, however, was not statistically significant ($p = 0.085$ and $p = 0.348$, respectively).

The concomitant daily dose of prednisolone for the responders was 2.56 ± 3.09 mg/day, for partial responders 2.39

± 2.64 mg/day and for non-responders 6.14 ± 3.60 mg/day. The difference in dose between responders and non-responders and between partial responders and non-responders was statistically significant ($p = 0.001$ and $p = 0.001$ respectively).

The duration of treatment with MTX was 20.04 ± 15.83 months (range 2 - 65 months) in the responders, 13.10 ± 8.41 (range 2 - 30 months) in the partial responders and 19.09 ± 11.78 (range 2 - 33 months) in the non-responders. There was no significant difference in the duration of treatment between responders versus partial responders ($p = 0.081$), responders versus non-responders ($p = 0.899$), or partial responders versus non-responders ($p = 0.168$).

The mean ESR and CRP for the whole group were 17.6 ± 15.0 mm/h and 7.3 ± 8.8 mg/L, respectively. The WCC, PMN and lymphocyte counts, platelet counts, Hb, serum albumin, red cell folate and MCV were $8.2 \pm 2.6 \times 10^9/L$, $5.6 \pm 2.4 \times 10^9/L$, $1.9 \pm 0.7 \times 10^9/L$, $273.7 \pm 61.5 \times 10^9/L$, 13.5 ± 1.5 g/dl, 41.3 ± 3.1 g/L, 751.9 ± 355.9 nmol/L and 88.3 ± 7.3 fL respectively.

Table II gives the results of blood counts and measurements of acute phase reactants in relation to response. The ESR and CRP were significantly lower in the responders versus the non-responders ($p = 0.007$, $p = 0.05$, respectively); however the MCV was significantly higher in the responders when compared to non-responders ($p = 0.047$). PMN counts in the responders were significantly lower than in both the partial responders and non-responders ($p = 0.0094$ and $p = 0.05$

Table II. Results of acute phase reactants and blood counts, including red cell indices and albumin, estimated at the time of classification of the patients according to their response to treatment with MTX.

Physician's global assessment	ESR Westergren (mm/ hr)	CRP (mg/L)	Hb (g/dL)	Total WCC $\times 10^9$ cells/L	PMN counts $\times 10^9$ cells/L	Lymphocyte counts $\times 10^9$ cells/L	Platelet counts $\times 10^9/L$	MCV (fL)	Red cell folate (nmol/L)	Albumin (g/L)
Responders	15.8 ± 14.1 (n = 56)	4.6 ± 3.7 (n = 49)	13.6 ± 1.5 (n = 56)	7.4 ± 2.3 (n = 56)	5.0 ± 2.3 (n = 43)	1.8 ± 0.6 (n = 45)	261.1 ± 54.0 (n = 56)	89.3 ± 8.0 (n = 56)	717.7 ± 359.3 (n = 44)	41.3 ± 3.1 (n = 54)
Partial responders	16.1 ± 10.5 (n = 29)	11.2 ± 12.3 (n = 26)	13.5 ± 1.4 (n = 29)	8.8 ± 2.1 (n = 29)	6.4 ± 1.9 (n = 20)	2.2 ± 0.8 (n = 17)	285.5 ± 60.8 (n = 29)	87.7 ± 8.8 (n = 29)	851.1 ± 393.0 (n = 21)	41.1 ± 3.1 (n = 28)
Non- responders	31.1 ± 22.4 (n = 11)	13.2 ± 12.1 (n = 6)	12.9 ± 1.4 (n = 11)	8.9 ± 3.3 (n = 11)	7.1 ± 3.0 (n = 7)	2.2 ± 0.7 (n = 7)	304.4 ± 83.1 (n = 11)	86.2 ± 5.6 (n = 11)	670.0 ± 86.9 (n = 7)	41.9 ± 3.5 (n = 10)

Table III. Intracellular MTX polyglutamate levels in patients receiving weekly doses of 2.5 to 37.5 mg for rheumatoid arthritis. The blood was taken at the time each patient was seen and classified according to his/her response to treatment with methotrexate.

Response category	E- MTX ($\mu\text{g/L}$ RBC)	MNC- MTX ($\text{pmol}/10^9$ cells)	PMN - MTX ($\text{pmol}/10^9$ cells)
Responders	27.69 ± 8.68 (n = 59)	203.38 ± 320.04 (n = 47)	300.12 ± 326.08 (n = 43)
Partial responders	23.15 ± 10.62 (n = 29)	107.97 ± 145.96 (n = 17)	167.11 ± 214.35 (n = 20)
Non-responders	9.76 ± 4.85 (n = 11)	85.14 ± 138.02 (n = 7)	31.16 ± 48.53 (n = 7)

$1\mu\text{g/L} = 2.2 \text{ nmol/L}$

respectively). Similarly, responders had significantly lower lymphocyte counts than partial responders ($p = 0.032$), but this difference did not reach statistical significance when compared with the non-responders ($p = 0.07$). Table II also gives the results for haemoglobin, WCC, platelet counts, red cell folate and serum albumin. None of these measurements differed significantly between groups.

Intracellular methotrexate polyglutamate

The intracellular MTX polyglutamate levels in erythrocytes (E-MTX $\mu\text{g/L}$ RBC), mononuclear cells (MNC-MTX $\text{pmol}/10^9$ cells) and polymorphs (PMN-MTX $\text{pmol}/10^9$ cells) for the three groups relative to the response are given in Table III. The number of estimations for red cell MTX totalled 99, but fewer measurements were done for the MNC ($n = 71$) and PMN ($n=70$) due to the difficulty experienced in separating these cells clearly in some of the samples.

Intracellular MTX polyglutamate concentrations in the RBC of responders and partial responders were significantly higher ($p = 0.000$) than in the non-responders. Similarly, both responders and partial responders had higher levels in the PMN than the non-responders, but this difference was only significant for the responders ($p = 0.0019$). A similar trend was seen in the concentrations in the MNC, but these were not significantly different between the groups. E-MTX was higher in patients with normal MCV ($80 - 96$ fl) than in those with low MCV (< 80 fl) (25.10 ± 10.84 versus 16.92 ± 6.97 $\mu\text{g/L}$ RBC respectively)

($p = 0.03$). Similarly, E-MTX was higher in patients with above normal MCV (> 96 fl) than in those with below normal MCV (< 80 fl) (25.0 ± 8.77 versus 16.92 ± 6.97 $\mu\text{g/L}$ RBC respectively) ($p = 0.046$) (Fig. 1).

Linear regression analysis showed no significant correlations between E-MTX and the ESR ($p = 0.633$), CRP ($p = 0.357$), Hb ($p = 0.947$), MCV ($p = 0.742$), red cell folate ($p = 0.478$) or liver enzymes, GT ($p = 0.056$), AST ($p = 0.882$), or ALP ($p = 0.117$). Similarly, there was no correlation between E-MTX and the PMN counts ($p = 0.814$), between the PMN-MTX concentration and the PMN counts ($p = 0.118$), or between the MNC-MTX concentration and either the total WCC ($p = 0.438$) or the lymphocyte counts ($p = 0.717$).

There was, however, a significant correlation between the PMN-MTX concen-

tration and the total WCC ($r = 0.243$, $p = 0.048$) (Fig. 2). Analysis of these results without the single significant outlier which was present (one patient had a much higher PMN-MTX concentration) yielded similar correlations ($r = 0.235$, $p = 0.059$).

Linear regression analysis of E-MTX in relation to the weekly dose of MTX without the outlier (one patient was on a much higher dose of MTX) showed no correlation between the MTX dose and E-MTX ($r = 0.049$, $p = 0.635$). However, there was a trend towards a positive correlation between E-MTX levels in responders and the weekly dose of MTX, although this did not reach significance ($r = 0.217$, $p = 0.086$) (Fig. 3).

There was no correlation between the weekly dose of MTX and PMN-MTX ($r = 0.175$, $p = 0.109$) or MNC-MTX ($r = 0.107$, $p = 0.377$).

Side effects

Side effects since the initiation of MTX were recorded in 13 of the 58 responders and included sore lips (2 patients), mouth ulcers (1 patient), nausea/vomiting (2 patients), hair loss (1 patient) and abnormalities of liver function necessitating dosage reduction or cessation (7 patients).

Of the 28 partial responders, 11 developed side effects including nausea in one, mouth ulcers in 3, nausea/mouth ulcers in 2, and abnormalities of liver function in 5. Out of 11 non-responders only one developed side effects (sore lips). The

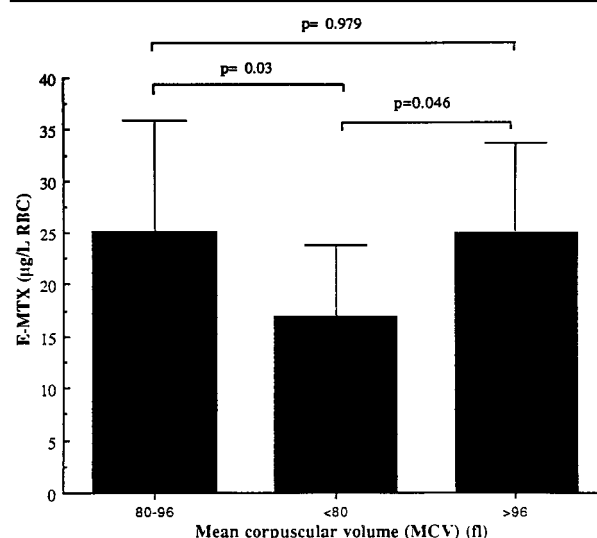


Fig. 1. E-MTX levels in patients with either normal MCV ($80 - 96$ fl), below normal MCV (< 80 fl), or above normal MCV (> 96 fl).

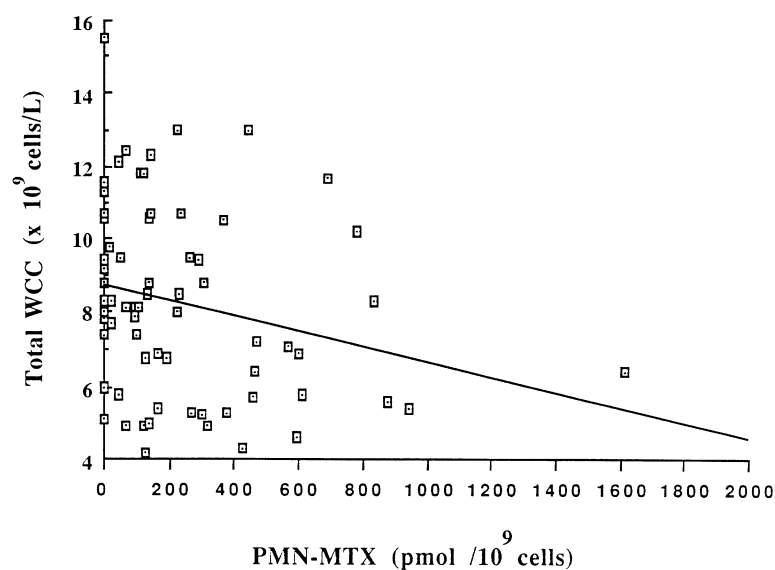


Fig. 2. Scattergram of the mean white cell counts in relation to the levels of PMN-MTX ($r = -0.243$, $p = 0.048$).

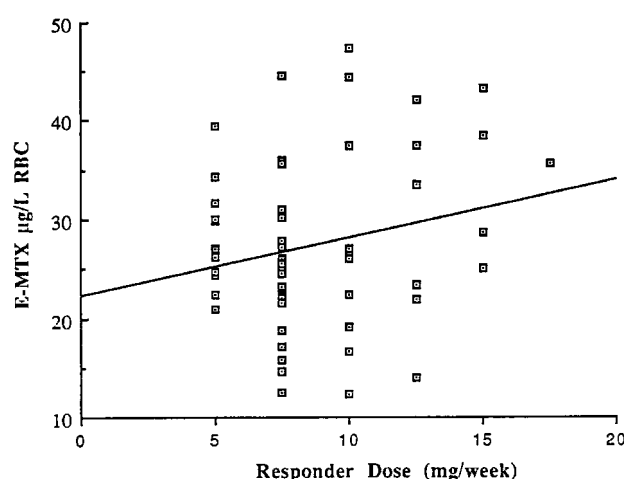


Fig. 3. E-MTX levels in responders in relation to the weekly dose of MTX, without the outlier ($r = 0.217$, $p = 0.086$).

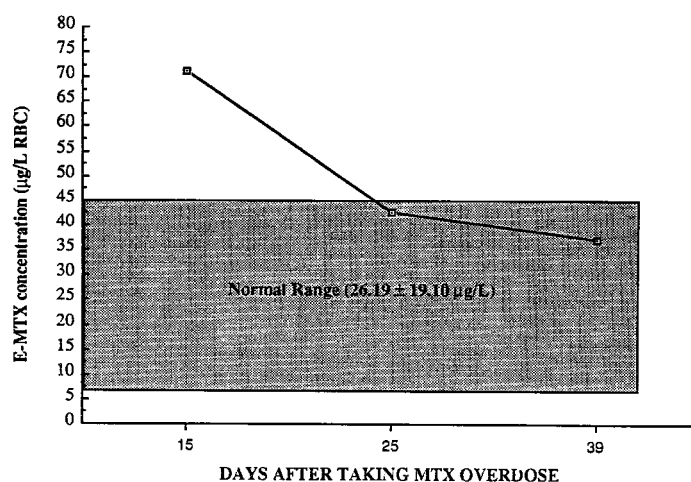


Fig. 4. E-MTX concentrations in one patient who took an overdose, having misunderstood our instructions. She developed severe mucositis requiring hospital admission, i.v. fluids and folic acid rescue. Oral methotrexate was re-started on day 34 at a weekly dose of 2.5 mg. The "normal" range (mean \pm 2 SD) given is that obtained from the responders and partial responders.

rate of development of side effects in the responders and partial responders compared to the non-responders was not significantly different ($\chi^2 = 4.615$, $df = 2$, $p = 0.10$).

The intracellular concentrations of MTX polyglutamates in patients with or without side effects attributed to MTX were also compared. There was no significant difference in the levels of E-MTX ($p = 0.15$), PMN-MTX ($p = 0.44$) or MNC-MTX ($p = 0.39$) in patients with or without side effects.

One patient who was unclassified and therefore not included in the above analyses, misunderstood a dosage change, and took too high a dose of MTX. This necessitated hospital admission due to severe oral mucositis, abdominal pain and diarrhoea requiring treatment with parenteral fluids and folic acid. MTX was re-started after full recovery, on day 34, at a weekly oral dose of 2.5 mg.

Intracellular MTX polyglutamate levels on days 15, 25 and 39 post-dose were 71.20, 42.68 and 37.12 $\mu\text{g/L}$ RBC (156.64, 93.90 and 81.66 nmoles/L RBC) in erythrocytes (E-MTX); 461.9, 102.65, 263.97 $\text{pmol}/10^9$ cells in mononuclear cells (MNC-MTX); and 318.96, 109.99 and 0 $\mu\text{g/L}$ $\text{pmol}/10^9$ cells in polymorphs (PMN-MTX), respectively. E-MTX in the toxic patient were above the mean + 2 SD of the E-MTX concentration (26.19 ± 19.10 $\mu\text{g/L}$) of the responder group as a whole (responders plus partial responders) two weeks after the overdose (Fig. 4). Her levels of PMN-MTX and MNC-MTX were always within the range observed in the rest of the patients.

Discussion

Our findings show that in rheumatoid arthritis, methotrexate polyglutamate levels in the erythrocytes of patients who were assessed as having responded or partially responded to the drug were significantly higher than in patients who were assessed as having failed to respond. There was a weaker correlation with the levels in polymorphs and no correlation with the levels in mononuclear cells. The poor correlation with polymorphs as opposed to erythrocytes may be due to the more rapid turnover of polymorphs, which have a half-life of

about 8 hours (23) compared to the erythrocytes which remain in the circulation for 2 to 3 months (15). The relatively weak correlation with polymorphs may also be due to the differences in proliferation and maturation times of the myeloid bone marrow cells, which are known to vary widely (24).

Although more stringent criteria for the response rates could have been used, (e.g., the ACR response criteria) treatment decisions in clinical medicine, particularly in patients with RA, are usually made based on global clinical assessments. In our study the physician's evaluation was based on clinical assessments which included a patient history, joint examination and a comparison with an earlier assessment of the patient's condition. In addition, the stratification of the subjects into their respective groups was made at the time of the study by the same treating rheumatologist to eliminate observer bias.

In childhood ALL, Schmiegelow *et al.* (19) showed that the MTX polyglutamate levels in erythrocytes correlate with the clinical response and prognosis. Methotrexate polyglutamate levels in erythrocytes were found to be a predictor of relapse, with higher concentrations being associated with a better prognosis.

In some respects the correlation of MTX polyglutamates in the red cells with the clinical response in RA and childhood ALL resembles the correlation of HbA_{1c} in red cells with long term glycaemic control in diabetes. Just as HbA_{1c} reflects red cell exposure to plasma glucose, E-MTX reflects bone marrow exposure to serum MTX. Since MTX is incorporated into red cell precursors (erythroblasts) in the bone marrow (25), it has been suggested that the erythrocyte MTX concentration reflects bone marrow drug exposure, as well as the ability of the RBC line to form polyglutamates (15, 26, 27) since only immature cells polyglutamate folic acid or MTX.

In mammalian cells, especially in the circulating erythrocytes and to a lesser extent in the circulating leucocytes, MTX is stored in the form of polyglutamate derivatives which are both active and quite long-lived. Methotrexate polyglutamates, particularly those with more

than three glutamyl residues, are retained in the cells longer than MTX, thus providing a mechanism by which MTX polyglutamates produce delayed or prolonged effects (28-31).

MTX-polyglutamates inhibit not only dihydrofolate reductase (10), but also other folate-requiring enzymes that are not substantially inhibited by MTX, including thymidylate synthase (32) and the transformylases required for *de novo* purine synthesis (33, 34). Studies performed by Allegra and coworkers (33), and subsequently by Baggott *et al.* (34) demonstrated that the enzyme that was inhibited most effectively by MTX polyglutamates was 5-aminoimidazole-4-carboxamide ribotide (AICAR) transformylase. A blockage at this step in the pathway of purine-nucleotide biosynthesis leads to the intracellular accumulation of 5-aminoimidazole-4-carboxamide (AICA) riboside and ribotide. Since AICA riboside directly inhibits adenosine deaminase (ADA) and AICA ribotide inhibits adenosine monophosphate (AMP) deaminase, the intracellular accumulation of these compounds could lead to the respective release of adenosine or AMP (which is converted by 5'-nucleotidase to adenosine) into the extracellular space (34). Adenosine is thought to mediate at least some of the anti-inflammatory effects of low-dose MTX (35).

A recent publication by Baggott *et al.* (36) showed a strong correlation between urinary AICA and AICAR levels and the response to MTX in adjuvant arthritis. This is consistent with prior proposals that the accumulation of intracellular AICAR metabolites leads to the therapeutic effects of MTX (35, 37).

Both MTX and MTX polyglutamates also inhibit a number of intracellular folate-dependent reactions, including the methylation of homocysteine to methionine. The conversion of homocysteine to methionine is required for the generation of S-adenosyl-methionine, which acts as the proximal methyl donor to RNA, DNA, proteins and phospholipids, as well as in the synthesis of polyamines. Inhibition of these transmethylation reactions by MTX may inhibit the cellular synthesis of polyamines which accumulate in the synovium of patients with RA

and which may contribute to joint destruction in this disease (35).

During treatment with MTX for RA, MTX polyglutamates accumulate intracellularly in the erythrocytes, MNC and PMN with large inter-individual variations at any particular dose. Several reasons could underlie this observed variability between patients, including variable compliance, variable absorption or metabolism, and a hepatic first-pass effect. Nevertheless, in our study the levels in responders and partial responders were significantly higher than in those who failed to respond. The differences in the circulating intracellular levels of MTX polyglutamates in the different responder groups could not be explained by the MTX dose or by the duration of treatment with MTX. In fact, there was a trend for the dose of MTX to be higher and for the duration of treatment to be shorter in non-responders. The concomitant prednisolone dose was significantly lower in the responders than in the non-responding group, further confirming the relationship between disease suppression and E-MTX. Similarly, the acute phase reactants correlated with the clinical assessment and response to the drug.

Linear regression analysis showed no correlation between the weekly dose of MTX and E-MTX. However, since the doses used in this study were quite low, especially in the non-responders, it is possible that with higher weekly dosing levels a more clear relationship between E-MTX and dose could emerge. The correlation of E-MTX levels with the mean corpuscular volume is consistent with the anti-folate effect of MTX and its polyglutamates and resembles that seen with sulphasalazine.

Since MTX polyglutamates are incorporated into the RBCs in the bone marrow, there may be a time delay between E-MTX levels and adverse effects. Any toxicity after beginning MTX treatment therefore has to be considered, and not merely the toxicity at the time of the assessment, which may be months after the incorporation of the MTX polyglutamates. No significant correlation with side effects was found, although there was a trend for levels to be higher in patients with side effects. One patient

who accidentally took too much MTX had E-MTX levels almost twice the upper limit observed in the rest of the patients. She developed severe life-threatening mucositis.

The measurement of E-MTX levels in patients with rheumatoid arthritis potentially offers a method for monitoring their response to MTX. Even in the presence of large inter-individual variations, a wide therapeutic range may still be defined. If the individual under good disease control consistently exhibits E-MTX levels in the upper region of the therapeutic range, this patient may benefit from drug concentration measurements, as a fall to the lower region may be associated with impaired control. Conversely, a well controlled patient at the lower end of the spectrum may exhibit toxicity when his E-MTX concentration rises. Thus, each patient may show a desirable narrow therapeutic range applicable to him/her as an individual, but this range for the group as a whole may be wide. Non-compliance may also be detected and levels beyond the upper limit may also be of value.

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